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Mitochondrial "kiss-and-run": Interplay between mitochondrial motility and fusion-fission dynamics

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   10 June 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. You will be pleased to see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address or respond to the referees' criticisms in an adequate manner.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this article Liu and coworkers describe in detail transient fusion of mitochondria, a phenomenon

they christen 'kiss-and-run' behavior. Authors provide an elegant description using PEG fusion assay and expression of fluorescent and photoactivable proteins. It should be stressed that this is the first report giving a detailed description of transient fusion. Authors divide fusion events in two classes (transient and complete) on the basis of the spatial location of the following fission event. The most noteworthy conclusion of this part of the paper is that transient fusion, even if on average significantly shorter than the complete fusion, enables effective bilateral exchange of components of IMS and mitochondrial matrix. Therefore 'kiss-and-run' sustain homogenous mixing of mitochondrial material and could prevent accumulation of dysfunctional mitochondria. The second part of the article provides clues about the mechanistic differences between transient and complete fusion. Authors observed the localization of Drp1 at the site of fusion almost simultaneously to the fusion event. After complete fusion, Drp1 leaves the site immediately, while in the 'kiss-and-run' it remains, leading to the following fission. This finding is of particular importance because it indicates a direct interplay between elements of fusion and fission machinery, opening an avenue of future research. It has been published that Drp1 colocalizes with Mfn2 at mitochondrial foci (Karbowski et al 2002) and that Mfn2 knockout increases Drp1 translocation to mitochondria (Parra et al 2008). However, the present manuscript reports for the first time the occurrence of Drp1 at sites of fusion. The influence of the fusion machinery on Drp1 localization and activity is further stresses by the observation that genetic manipulation of Opa1 levels differently regulates transient and complete fusion. Authors also focus on the relationship between movement of mitochondria and their fusion. Frontal interaction, e.g. between mitochondria transported along single microtubule in opposite directions, favors complete fusion, while side-by-side contact results in transient fusion. This novel observation supports an emerging possibility, that physical force provided by transport machinery may be a prerequisite for mitochondrial fission (Pukadyil and Schmidt, 2008 Bowes and Gupta, 2008). In the last part of the paper authors verify relationship between the mode of fusion and bioenergetic proprieties of mitochondria, and conclude that preservation of transient fusion enables support of respiration.

Summarizing, the paper provides the reader with the first detailed description of transient fusion, proposes mechanistic explanations of the observed phenomenon and insights into its physiological relevance. There are only a few minor concerns:

1. Mitofusins are not doing the same job in mitochondrial fusion. There is large evidence for a differential role of Mfn1 vs. Mfn2, which is anyway involved in other functions ranging from metabolic control (Bach et al) to tethering to ER (Brito and Scorrano). Please change the introduction accordingly

2. Authors suggest that cells overexpressing Opa1 display a higher electrochemical potential and conclude that transient fusion 'is competent to maintain mitochondrial metabolism and bioenergetics'. Care should be taken to exclude possible artifacts. Higher accumulation of potentiometric dyes could be the consequence of the ATP synthase working in reversal, or of changes in plasma membrane potential. The former possibility is partially excluded by showing that respiratory chain is fully active in Opa1 overexpressing cell. Nevertheless, this control is not fully relevant, as it was performed in a different cell line. Authors should check whether oligomycin and incubation in depolarizing concentrations of KCl affect membrane potential

3. Authors test relationship between mitochondria movements and fusion of mitochondria using agents which affect cytoskeleton (nocodazole) or cause robust increase in calcium signaling (vasopressin). Both stimuli are quite unspecific and may influence phenomenon in question by mechanisms other than mobility of mitochondria, e.g. Drp1 localization on mitochondria depends on calcium signaling (Cereghetti et al, PNAS 2008, which should be cited in the discussion on the role of Ca2+ in affecting transient fission). In this context it would be appropriate to use a more specific approach, like genetic manipulation of Miro expression, already published by the same group (Satome et al, 2008).

Referee #2 (Remarks to the Author):

Liu et al. discovered different modes of fusion between mitochondria by carefully examining the kinetics of fusion with fluorescence microscopy. The classical mode consists of two mitochondria approaching each other along the same mitocrotubule, followed by fusion at their tips and complete

exchange of contents. In contrast, the new mode consists of one mitochondrion approaching another one on the side, followed by transient fusion, exchange of soluble contents, quickly followed by fission and withdrawal of the fusing mitochondrion. The evidence for these distinctions is provided by tracking individual fusion events with various forms of photo-activatable GFP fused to mitochondrial intermembrane space, inner membrane and matrix proteins. The authors were able to observe a delay between mixture of IMS and matrix contents, suggesting that fusion of mitochondrial outer membranes precedes that of inner membranes by 2.4 sec on average. They also investigated the effects of different levels of Opa1 expression on the two types of fusion and their dependence on mitochondrial membrane potential, on Drp1 and on microtubules.

In general, I (found? think?) this was of general interest. It describes different kinds of fusion and it explores some of the parameters involved in their differences. Although it relies heavily on microscopy, the evidence for differences is convincing. I have one basic quibble. The two kinds of fusion seem to be different manifestations of the same process with variations determined by the angles or force with which mitochondria are driven together, rather than by fundamental differences in fusion and fission machineries. In that sense, I think it is different from kiss and run at the plasma membrane where one process involves coated vesicle formation and the other one does not. I would make this distinction in the discussion (conclusion) section.

Some other points need to be addressed:

The discovery that fusion still occurs after dissipation of membrane potential contradicts results obtained previously with yeast (yeast mitochondria fail to fuse without membrane potential even though they have no mechanism for membrane potential dependent inactivation of Mgm1). I worry about the effectiveness of FCCP in this experiment. FCCP induced cleavage of Opa1 does not seem to be as complete as that observed in papers from other labs, which makes me worry that there may not have been enough FCCP for complete inhibition of fusion.

The Opa1 overexpression experiments show very high levels, especially in adenovirus transfected cells, making it difficult to assign physiological relevance to these results.

Some of the data were not as strong as one would like to see. I had trouble deciding whether some of the results were statistically significant. Tests should be provided where possible. Specifically the shifts between fusion types observed with various treatments: Opa1, drp1, delta psi, etc.

The writing could be a lot tighter. Some sentences are real clunkers. Examples: the first few sentence on the role of drp1 are muddled by switching back and forth between in fission or reverse fusion and the first sentence of the conclusions section is grammatically incorrect. The section on membrane potential needs to be written more clearly, preferably in multiple paragraphs, instead of one page long paragraph.

Referee #3 (Remarks to the Author):

In this study, Liu and coworkers perform a thorough analysis of mitochondrial fusion in real time. The study is very well performed and makes use of precise and potent fluorescence microscopy approaches, notably photoactivatable paGFP and photoconvertible KFP. The authors achieve a very high temporal and spatial resolution that results in the most detailed dissection of the mitochondrial fusion process achieved to date.

Authors show that sudden dissipation of mitochondrial deltaPSIm does not result in the immediate inhibition of IMM fusion, suggesting that mitochondrial fusion does not directly depend on mitochondrial deltaPSI, but on downstream, deltaPSI-dependent factors. This is a highly significant finding in the field.

With their novel tools in hand, the authors describe two types of fusion: complete fusion of mitochondria, leading to the formation of "new" merged mitochondria and transient "kiss and run" fusion, where independent mitochondria merge their outer and inner membranes transiently, exchange some of their contents and separate again while maintaining their original shape.

The article is then devoted to the characterization of the molecular, cellular and energetic requirements of these two classes of fusion. The predominance of one or the other fusion type is shown to be modulated by dynamin-related proteins involved in fusion (OPA1) and fission (DLP1), by the relative position and mobility of the mitochondria destined to fuse, and by mitochondrial bioenergetics. This study reveals a new, previously unknown phenomenon ("kiss and run" fusion) and significantly improves our knowledge of the mitochondrial fusion process.

The following points need to be adressed:

Major points:
1. Authors propose that mitochondria can engage in one OR the other class of fusion, and that the class of fusion is determined by OPA1 levels (1), relative position/movement (2) and mitochondrial bioenergetics (3).
To my opinion, another model is possible to explain these findings.
Mitochondria can engage successively in (1) outer membrane fusion, (2) kiss IMM fusion (short membrane merge w/o loss of original morphology) and (3) complete fusion. Authors clearly show that, surprisingly, very few fusion processes are aborted after step 1. However, a significant proportion of fusions are aborted after "kiss" step 2 (kiss and run) and only 10-20% of fusions complete step 3 (kiss and stay). The Factors (1) to (3) may modulate the reaction leading from step 2 to step 3.
Note that the increase in the frequency of complete fusion by NCD (page 13) is presented by the authors as a preferential inhibition of transient fusion. In the alternative sequential model, the lack of movement (after the kiss) induced by NCD would favour the "completion" of the fusion process (kiss and stay).

The model proposed by authors is valid. However, authors need to argue (in their answer and in the manuscript) why they think that their data exclude the alternative, sequential model.

2. Effect of oligomycin on mitochondrial bioenergetics (page 15). This part of the manuscript contains erroneous analysis and interpretations that need to be corrected.

It is well established that oligomycin inhibits the ATPase/F1 domain of the ATP-synthase (both "forward" ATP synthesis and "reverse" ATP-hydrolisis). Inhibition of the ATP-synthase with oligomycin blocks the passage of protons through the F0 domain. In coupled respiring mitochondria, this leads to no changes in the deltaPSI or to a slight hyperpolarization that inhibits electron transport through the respiratory chain (this is THE coupling between electron transport and ATP synthesis).
Upon inhibition of electron transport (cyanide, antimycin, rotenone), the ATPase hydrolizes ATP and pumps protons to maintain the deltaPSI. Thus, oligomycin only leads to loss of deltaPSI if inhibitors of the respiratory chain (cyanide, antimycin, rotenone) are added simultaneously.
The slight hyperpolarization of mitochondria by oligomycin has been established by several authors since the 1960s (but is also reported in more recent papers in te field of mitochondrial dynamics, e.g Legros et al 2002). It can be also observed in the reference cited by the authors (Ward et al 2000, Fig. 4).

These facts are incompatible with several confusing statements and conclusions from the authors that need to be corrected.
- page 15: "oligomycin (...) causes depolarization of the mitochondria that use cytoplasmic ATP to maintain the deltaPSI through reverse ATP synthase (...) but evokes hyperpolarization in the ones that produce ATP at the expense of their deltaPSI."
Is the ATPase activity inhibited or not? How can an oligomycin-inhibited ATP-synthase hydrolize cytoplasmic ATP or produce ATP?
- page 8:
top: a combination of oligomycin and FCCP is designed as "uncoupler".
bottom: "oligomycin was added to slow down the uncoupling induced ATP depletion".

Minor points:
1. What is the relationship between transient fusion and the fast succession of fusion-fission previously described by Twig et al (2008)?
2. The nature of the IMM protein used in this study deserves a better description. Available studies

with PEG fusion have analyzed the dynamics of respiratory complex IV (Legros 2004) or complex I (Busch 2006). What are the size and properties of the GFP-tagged transporter used in this study?

3. Page 8: authors state "processing of OPA1 to a form that is not competent to support fusion". The functional model proposed by Reichert et al (short OPA1 is not competent for fusion) was chanllenged by Song et al (2007) who showed that fusion competent mitochondria require short AND long OPA1.

4. Page 8/9: "The probability (...) in FCCP-pretreated cells. How long was the pre-treatment?

5. Page 11: Drp1K38A. Other authors (James 2003, Pitts 1999) show that Drp1K38A forms clusters in the cytosol. The divergent result should be commented.

6. Page 12, SFig3A: Alignment of microtubules and mitochondria is only seen after significant enlargement. Authors may show an enlargement of a selected area.

7. Page 16, first paragraph. If I understood right, authors analyzed control (non transfected) cells with fragmented mitochondria. They found that their mitochondria had a lower deltaPSIm than those of OPA1 overexpressing cells.

It would be interesting to know whether control cells with fragmented mitochondria also had a lower deltaPSIm than control cells with elongated mitochondria. This may explain the shift of the fusion/fission equilibrium towards fission.

1st Revision - authors' response   17 July 2009

Referee #1 (Remarks to the Author):

We greatly appreciate the Reviewer's thoughtful suggestions and positive comments.

There are only a few minor concerns:

1. Mitofusins are not doing the same job in mitochondrial fusion. There is large evidence for a differential role of Mfn1 vs. Mfn2, which is anyway involved in other functions ranging from metabolic control (Bach et al) to tethering to ER (Brito and Scorrano). Please change the introduction accordingly.

We have changed the text as suggested (page3 para2 line6-7).

2. Authors suggest that cells overexpressing Opa1 display a higher electrochemical potential and conclude that transient fusion 'is competent to maintain mitochondrial metabolism and bioenergetics'. Care should be taken to exclude possible artifacts. Higher accumulation of potentiometric dyes could be the consequence of the ATP synthase working in reversal, or of changes in plasma membrane potential. The former possibility is partially excluded by showing that respiratory chain is fully active in Opa1 overexpressing cell. Nevertheless, this control is not fully relevant, as it was performed in a different cell line. Authors should check whether oligomycin and incubation in depolarizing concentrations of KCl affect membrane potential

TMRE fluorescence in the Opa1 overexpressing cells showing mitochondrial fragmentation ("Opa1 overexpressing") was compared to that in both generic mock-transfected ("control") and mitochondrial fragmentation showing mock-transfected cells ("fragmented control"). Fig7B shows that the apparent mitochondrial density is higher in the Opa1-overexpressing cells than in the control. Since this difference might have caused overestimation of the TMRE fluorescence (cell fluorescence/cell area) we used an algorithm to remove the dark pixels and recalculated the TMRE fluorescence (mito fluorescence/mito area). After this correction, the TMRE signal in the Opa1 overexpressing cells is 111±16% of the signal in the control cells (n=12, Fig7D and revised text page16 para1 line6). Thus, the $\Delta\Psi$m seems to be similar in both control and high level of Opa1 overexpressing cells. By contrast, the fragmented control population had greatly reduced m (TMRE fluorescence was 34±5% of the control, n=5, Fig7D). We appreciate the Reviewer's comment that helped us to clarify this point.

Oligomycin elicited only a minor change in the TMRE signal in naive cells (SupplFig4A), whereas KCl (60mM) that evokes rapid plasma membrane depolarization (Szalai et al 2000) caused only a slow and small decrease in the TMRE fluorescence. Thus, it is unlikely that an effect of Opa1 on the

F1F0 ATPase activity or on the plasma membrane potential would affect the TMRE signal.

3. Authors test relationship between mitochondria movements and fusion of mitochondria using agents which affect cytoskeleton (nocodazole) or cause robust increase in calcium signaling (vasopressin). Both stimuli are quite unspecific and may influence phenomenon in question by mechanisms other than mobility of mitochondria, e.g. Drp1 localization on mitochondria depends on calcium signaling (Cereghetti et al, PNAS 2008, which should be cited in the discussion on the role of Ca2+ in affecting transient fission). In this context it would be appropriate to use a more specific approach, like genetic manipulation of Miro expression, already published by the same group (Satome et al, 2008).

We also thought about targeting of Miro as a means to influence motility in a more specific manner. However, both biochemical and functional studies indicate that Miro may directly interact with the fusion and fission machinery (Saotome et al 2008, Weihofen et al 2009 or a recent review Liu & Hajnoczky). Therefore, targeting of Miro does not seem to offer a particularly useful approach.

Regarding the vasopressin effect, we did not notice obvious mitochondrial fragmentation during the cytoplasmic [Ca2+] spikes in H9c2 cells. However, in agreement with Cereghetti et al. 2008, mitochondrial fragmentation occurred during sustained [Ca2+] elevations that can be attributed to the Drp1 translocation to the mitochondria. We included citation of the Cereghetti et al, PNAS 2008 paper as suggested (page14 para2 line15-17).

Referee #2 (Remarks to the Author):

We would like to thank the reviewer for the insightful suggestions and positive comments.

I have one basic quibble. The two kinds of fusion seem to be different manifestations of the same process with variations determined by the angles or force with which mitochondria are driven together, rather than by fundamental differences in fusion and fission machineries. In that sense, I think it is different from kiss and run at the plasma membrane where one process involves coated vesicle formation and the other one does not. I would make this distinction in the discussion (conclusion) section.

This point is clarified in the Conclusions (page18 para1 line1-3): "However, kiss-and-run is discriminated from full-collapse exocytosis by the lack of clathrin coat (Rizzoli and Jahn, 2007), whereas mitochondrial kiss-and-run and complete fusion seem to involve common fusion proteins, though depend differently on Opa1 amount."
We also discuss in the revised text the possibility that IMM kiss and complete fusion are successive events and the Opa1 and cytoskeletal arrangement of the interacting mitochondria would determine whether the IMM kiss is followed by separation or complete fusion (page17 para1).

Some other points need to be addressed:
The discovery that fusion still occurs after dissipation of membrane potential contradicts results obtained previously with yeast (yeast mitochondria fail to fuse without membrane potential even though they have no mechanism for membrane potential dependent inactivation of Mgm1). I worry about the effectiveness of FCCP in this experiment. FCCP induced cleavage of Opa1 does not seem to be as complete as that observed in papers from other labs, which makes me worry that there may not have been enough FCCP for complete inhibition of fusion.

FCCP (5uM) caused rapid and complete release of both dyes DiIC1(5) and TMRE in the present work and in previous studies of H9c2 cells (eg Szalai et al 2000 J Biol Chem). An inherent limitation of the potentiometric probes is that the fluorescence measurements may fail to detect if a small fraction of the $\Delta\Psi m$ remains (eg a few tens of mVs out of -180mV). However, if the loss of fusion activity would depend on slow loss of a small remaining $\Delta\Psi m$ then the recovery of $\Delta\Psi m$ would be expected to rescue fusion activity. To test this point, cells were pretreated with FCCP and subsequently FCCP was washed out. The washout resulted in recovery of the m in <5 min, whereas the fusion activity showed <10% recovery (FCCP for 30min, washout 15min (n=3) or FCCP 60min,

washout 30min (n=6)). Collectively, these results indicate that dissipation of the ΔΨm causes loss of a fusion factor that is recovered with a delayed kinetic after restoration of the ΔΨm. Opa1 is a good candidate for this factor since its high molecular weight form is processed and recovered with a delayed kinetic after changes in the ΔΨm, but there can be another fusion factor that is processed in both yeast and mammalian cells. Nevertheless, a central role for Opa1 in H9c2 cells is supported by the fact that the uncoupler-induced fusion inhibition appeared much slower in Opa1-overexpressing than in control cells.

Regarding the kinetic and extent of the uncoupler-induced Opa1 processing, our result shows that FCCP needs 15min to result in maximal response. In some experiments, the high molecular weight band completely disappeared (immunoblot in the left and revised Fig3) or a small or a small fraction survived the FCCP treatment (Immunoblot in the right and original Fig3):

FCCP (5uM)-induced processing of the high molecular weight form of Opa1 in H9c2 cells
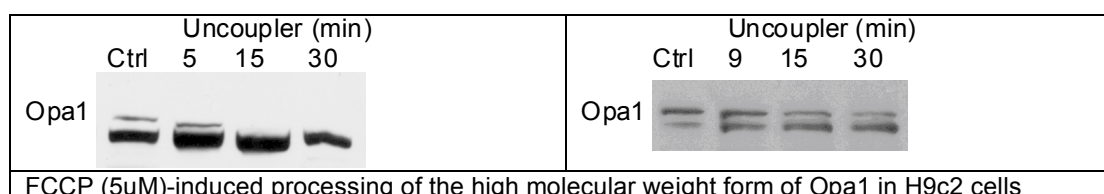
The observed kinetic and the extent of cleavage both seem to be consistent with published data obtained with supramaximal FCCP. Gradual loss (Griparic et al 2007 J Cell Biol; incomplete loss by 30min (Baricault et al 2007 Exp Cell Res); complete loss by 30min (Duvezin-Caubet et al 2006 J Biol Chem, Ishihara et al 2006 J Biol Chem, Guillery et al 2007 Biology of the Cell).

The Opa1 overexpression experiments show very high levels, especially in adenovirus transfected cells, making it difficult to assign physiological relevance to these results.

Different cell types show varying Opa1 expression levels and in some cases of Opa1 mutation associated with autosomal dominant optic atrophy, the expression level of Opa1 isoforms is increased (Cornille et al. 2008). Regardless, we agree with the Reviewer's point that massive overexpression that results from adenoviral Opa1 delivery is not expected to happen under physiological conditions. However, in the present study, this condition was useful to study the significance of transient fusion in isolation.

Some of the data were not as strong as one would like to see. I had trouble deciding whether some of the results were statistically significant. Tests should be provided where possible. Specifically the shifts between fusion types observed with various treatments: Opa1, drp1, delta psi, etc.

We have added statistical analysis to the results shown in Figs 4,5 and 7 as suggested. See revised figures. Furthermore, a table has been added (SuppTable1) to illustrate the changes in the frequency of transient fusions in relation to complete fusions.

The writing could be a lot tighter. Some sentences are real clunkers. Examples: the first few sentence on the role of drp1 are muddled by switching back and forth between in fission or reverse fusion and the first sentence of the conclusions section is grammatically incorrect. The section on membrane potential needs to be written more clearly, preferably in multiple paragraphs, instead of one page long paragraph.

The text has been rewritten to increase clarity (page11 para2). The section on membrane potential was revised and was separated into 3 paragraphs (page8-9).

Referee #3 (Remarks to the Author):

We are grateful for the helpful thoughts and for the positive comments.

1. Authors propose that mitochondria can engage in one OR the other class of fusion, and that the class of fusion is determined by OPA1 levels (1), relative position/movement (2) and mitochondrial bioenergetics (3).
To my opinion, another model is possible to explain these findings.
Mitochondria can engage successively in (1) outer membrane fusion, (2) kiss IMM fusion (short membrane merge w/o loss of original morphology) and (3) complete fusion. Authors clearly show that, surprisingly, very few fusion processes are aborted after step 1. However, a significant proportion of fusions are aborted after "kiss" step 2 (kiss and run) and only 10-20% of fusions complete step 3 (kiss and stay). The Factors (1) to (3) may modulate the reaction leading from step 2 to step 3.
Note that the increase in the frequency of complete fusion by NCD (page 13) is presented by the authors as a preferential inhibition of transient fusion. In the alternative sequential model, the lack of movement (after the kiss) induced by NCD would favour the "completion" of the fusion process (kiss and stay).
The model proposed by authors is valid. However, authors need to argue (in their answer and in the manuscript) why they think that their data exclude the alternative, sequential model.

The Reviewer's suggestion is well taken. There is no feature of the IMM kiss that is excluded from complete fusion and therefore it is entirely possible that kiss IMM fusion and complete fusion occur successively when the conditions are favorable for complete fusion. These conditions would include a particular stoichiometry among Opa1 molecules (and perhaps other factors) and the absence of mechanical tension at the fusion site. However, the Opa1 and cytoskeletal arrangement of the interacting mitochondria will pre-determine if the merge will be kiss-and-run or prevails to complete fusion. The text has been modified to include this possibility (Page17 Para1).

2. Effect of oligomycin on mitochondrial bioenergetics (page 15). This part of the manuscript contains erroneous analysis and interpretations that need to be corrected.
It is well established that oligomycin inhibits the ATPase/F1 domain of the ATP-synthase (both "forward" ATP synthesis and "reverse" ATP-hydrolisis). Inhibition of the ATP-synthase with oligomycin blocks the passage of protons through the F0 domain. In coupled respiring mitochondria, this leads to no changes in the deltaPSI or to a slight hyperpolarization that inhibits electron transport through the respiratory chain (this is THE coupling between electron transport and ATP synthesis).
Upon inhibition of electron transport (cyanide, antimycin, rotenone), the ATPase hydrolizes ATP and pumps protons to maintain the deltaPSI. Thus, oligomycin only leads to loss of deltaPSI if inhibitors of the respiratory chain (cyanide, antimycin, rotenone) are added simultaneously.
The slight hyperpolarization of mitochondria by oligomycin has been established by several authors since the 1960s (but is also reported in more recent papers in te field of mitochondrial dynamics, e.g Legros et al 2002). It can be also observed in the reference cited by the authors (Ward et al 2000, Fig. 4).

These facts are incompatible with several confusing statements and conclusions from the authors that need to be corrected.
- page 15: "oligomycin (...) causes depolarization of the mitochondria that use cytoplasmic ATP to maintain the deltaPSI through reverse ATP synthase (...) but evokes hyperpolarization in the ones that produce ATP at the expense of their deltaPSI."
Is the ATPase activity inhibited or not? How can an oligomycin-inhibited ATP-synthase hydrolize cytoplasmic ATP or produce ATP?

We revised in the text as follows:
"The contribution of transient fusions to the maintenance of the metabolic activity of the individual mitochondria was first tested by the ATP synthase inhibitor oligomycin. In healthy mitochondria that produce ATP at the expense of their $\Delta\Psi m$ oligomycin causes slight hyperpolarization. By contrast, in mitochondria that use cytoplasmic ATP to maintain the $\Delta\Psi m$ through a reverse ATP synthase activity oligomycin causes depolarization (Ward et al., 2000)." (page15 para2)

- page 8:
top: a combination of oligomycin and FCCP is designed as "uncoupler".
The sentence has been changed to clarify that FCCP is referred as uncoupler.

bottom: "oligomycin was added to slow down the uncoupling induced ATP depletion".
This statement was omitted from the revised text.

Minor points:
1. What is the relationship between transient fusion and the fast succession of fusion-fission previously described by Twig et al (2008)?

Both transient fusion and the fast succession of fusion-fission show rapid kinetics of mitochondrial merging and separation. Transient fusion shows preservation of the morphology of the interacting mitochondria at least, at the resolution of confocal microscopy. This aspect is difficult to assess in the study of Twig et al (2008), since most of the interacting mitochondria are globular. Since succession of fusion-fission yields segregation of waste material in one of the offsprings, it is likely to involve considerable mixing of the integral membrane components. Notably, the deltaPSIm does not seem to be altered during transient fusion, whereas fast succession of fusion and fission was found to cause increased and decreased polarization in the two offsprings. Thus, the fusion events described in Twig et al (2008) and in the present study share similar manifestation but would have complementary function.

2. The nature of the IMM protein used in this study deserves a better description. Available studies with PEG fusion have analyzed the dynamics of respiratory complex IV (Legros 2004) or complex I (Busch 2006). What are the size and properties of the GFP-tagged transporter used in this study?

 "IMM-PAGFP is the mitochondrial targeting sequence (105AA) of ABCB10 fused with PAGFP (Twig et al., 2006). ABCB10 is a mitochondrial inner membrane erythroid transporter involved in heme biosynthesis." This has been added to Experimental Procedures (page18 last para page19 para1).

3. Page 8: authors state "processing of OPA1 to a form that is not competent to support fusion". The functional model proposed by Reichert et al (short OPA1 is not competent for fusion) was chanllenged by Song et al (2007) who showed that fusion competent mitochondria require short AND long OPA1.

We revised in the text: "The recent discovery that dissipation of $\Delta\Psi_m$ facilitates the processing of Opa1 to a form that is not competent to support fusion by itself (Duvezin-Caubet et al., 2006; Ishihara et al., 2006 Song et al., 2007)" (page8 last para).

4. Page 8/9: "The probability (...) in FCCP-pretreated cells. How long was the pre-treatment?

FCCP-pretreatment was for (15 min) (page9 para1).

5. Page 11: Drp1K38A. Other authors (James 2003, Pitts 1999) show that Drp1K38A forms clusters in the cytosol. The divergent result should be commented.

Drp1K38A CFP showed fairly homogenous distribution in the present study. Clusters that have been observed in previous reports (James et al., 2003, Pitts et al., 1999) occurred in only few H9c2 cells. This difference is noted in the revised text (Page11 Para2).

6. Page 12, SFig3A: Alignment of microtubules and mitochondria is only seen after significant enlargement. Authors may show an enlargement of a selected area.

We revised the figure to show an enlargement of a selected area (Supplementary Fig3A).

7. Page 16, first paragraph. If I understood right, authors analyzed control (non transfected) cells with fragmented mitochondria. They found that their mitochondria had a lower deltaPSIm than those of OPA1 overexpressing cells.
It would be interesting to know whether control cells with fragmented mitochondria also had a

lower deltaPSIm than control cells with elongated mitochondria. This may explain the shift of the fusion/fission equilibrium towards fission.

deltaPSIm of control cells with fragmented mitochondria, and Opa1 overexpressing cells with fragmented mitochondria were normalized to the deltaPSIm in the non-transfected control cells which have normal mitochondria. The deltaPSIm was decreased in the control cells with fragmented mitochondria but it was sustained in the Opa1 overexpressing cells with fragmented mitochondria. Thus, the control cells with fragmented mitochondria have lesser deltaPSIm than the control cells with more elongated mitochondria. The text has been modified to clarify this point (page16 para1).

2nd Editorial Decision   29 July 2009

Thank you for sending us your revised manuscript. Our original referees 1 and 3 have now seen it again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal. Before this will happen I was wondering whether you would like to consider modifying the tile of the manuscript as suggested by referee 1. One suggestion would be a title like "Mitochondrial transient, kiss and run fusion is distinct from Opa1-dependent full fusion". If you would like to modify the title, please send us a modified manuscript text file via e-mail and we will upload it into the system for you. Thank you very much.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

Authors exhaustively addressed my comments. I just don't think that the title is explicatory enough and it should be changed for the sake of clarity.

Referee #3 (Remarks to the Author):

The authors have responded to all comments and questions from myself and from the other reviewers. The articles nicely presents and discusses novel and original results that significantly improve our knowledge of the mitochondrial fusion process.